FEBS Letters 585 (2011) 1979–1984

FEBS Letters
journal homepage: www.FEBSLetters.org

Lipin-1γ isoform is a novel lipid droplet-associated protein highly expressed in the brain

Huajin Wang [a,*,1], Jing Zhang [a], Wei Qiu [a], Gil-Soo Han [b], George M. Carman [b], Khosrow Adeli [a,*]

[a] Program in Molecular Structure and Function, Research Institute, The Hospital for Sick Children, University of Toronto, Toronto, Ontario, Canada M5G 1X8
[b] Department of Food Science and Rutgers Center for Lipid Research, Rutgers University, New Brunswick, NJ 08901, USA

ARTICLE INFO

Article history:
Received 11 April 2011
Revised 11 May 2011
Accepted 12 May 2011
Available online 20 May 2011

Edited by Sandro Sonnino

Keywords:
Lipin
Phosphatidic acid phosphatase
Lipid droplet
Brain

ABSTRACT

Lipin-1 proteins are phosphatidic acid phosphatases (PAPs) catalyzing the conversion from phosphatidic acid (PA) to diacylglycerol (DG). Two alternative splicing isoforms, lipin-1α and -1β, are localized at different subcellular compartments. A third splicing isoform, lipin-1γ was recently cloned and its subcellular localization is unknown. Here, we demonstrate that lipin-1γ is localized to lipid droplets (LDs), an association mediated by a hydrophobic, lipin-1γ-specific domain. Additional expression of lipin-1γ altered LD morphology without affecting the triacylglycerol (TG) level. In human tissues, lipin-1γ is the main lipin-1 isoform expressed in normal human brain, suggesting a specialized role in regulating brain lipid metabolism.

1. Introduction

A late step in triacylglycerol (TG) and phospholipid (PL) synthesis is the dephosphorylation of phosphatidic acid (PA) to yield diacylglycerol (DG), catalyzed by phosphatidic acid phosphatases (PAP). Lipin family proteins have been identified as the Mg^{2++}-dependent, type 1 PAP enzymes (PAP1) [1,2]. Humans and mice contain three members of the lipin protein family: lipin-1, lipin-2, and lipin-3. Lipin-1 is the most studied member and mutations in the corresponding gene are causal to a murine fatty liver dystrophy (*fld*) phenotype, characterized by lipodystrophy, insulin resistance, neonatal hypertriglyceridemia and fatty liver, as well as peripheral neuropathy [3,4]. Conversely, transgenic overexpression of lipin-1 in skeletal muscle or white adipose tissues promotes adiposity in mice [5]. Hepatic lipin-1 has been implicated in regulating TG-rich very-low density lipoprotein (VLDL) assembly and secretion [6,7], in accordance with its role in TG synthesis.

Adding to the complexity of PAP expression, alternative splicing of lipin-1 messages generates three isoforms in human and mouse: lipin-1α, lipin-1β, and the recently cloned lipin-1γ [8,9]. The primary sequence of lipin-1α contains 890 amino acid residues, while lipin-1β contains an additional β-specific region of 36 amino acids (242–277) and lipin-1γ contains a γ-specific region composed of 26 amino acids (536–561) [8]. All three isoforms have PAP1 activity, although the kinetics of lipin-1γ activity in a micelle system was much different from that of α- and β-isoforms [8], suggesting differential binding of these isoforms to membranes. Despite the presence of a putative nuclear localization signal (NLS) in all isoforms, lipin-1α and -1β are found in different subcellular compartments. Lipin-1 and PAP1 activity predominantly reside in the cytosol when phosphorylated by insulin signals, whereas when dephosphorylated by signals such as oleic acid (OA), the majority of the α-isoform is located in the nucleus to function as a transcriptional co-activator of lipid metabolism genes and the β-isoform becomes mainly associated with the ER membrane to carry out the PAP enzyme function [10–12]. Similar to the other isoforms, lipin-1γ also contains an NLS, however its subcellular localization is unknown. In this study, we report the unique localization of this new lipin-1 isoform on lipid droplets (LDs).

Abbreviations: ADRP, adipose differentiation-related protein; CNX, calnexin; DG, diacylglycerol; DGAT, diacylglycerol acyltransferase; *fld*, fatty liver dystrophy; LD, lipid droplet; MAM, mitochondria-associated membranes; NLS, nuclear localization signal; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; PL, phospholipid; TG, triacylglycerol; VLDL, very-low density lipoprotein

* Corresponding authors. Address: Division of Clinical Biochemistry, DPLM, The Hospital for Sick Children Toronto, Ontario, Canada M5G 1X8. Fax: +1 416 813 6257 (K. Adeli).

E-mail addresses: huajin.wnag@yale.edu (H. Wang), k.adeli@utoronto.ca (K. Adeli).

[1] Present address: Department of Cell Biology, Yale School of Medicine, 333 Cedar Street, SHM C431, New Haven, CT 06520, USA. Fax: +1 203 785 7446.

0014-5793/$36.00
doi:10.1016/j.febslet.2011.05.035

2. Materials and methods

2.1. Plasmids

cDNAs encoding human lipin-1α, β and γ were amplified by PCR from pGH322, pGH327, pGH321 [8], respectively and subcloned into pEGFP-N1 to generate EGFP fusion proteins. Truncation mutants of lipin-1γ were obtained by PCR using the full-length lipin-1γ as template and inserted into pEGFP-N1 vector to generate EGFP fusion proteins.

2.2. Immunofluorescent staining and confocal microscopy

Cells were seeded on coverslips and transiently transfected with plasmids encoding EGFP fusion proteins of lipin-1 isoforms and truncation mutants. To stain LDs, cells were incubated with 6 μM BODIPY 558/568 C_{12} (Invitrogen) in the presence or absence of OA/BSA and fixed with 4% paraformaldehyde in PBS before imaging. For immunofluorescent staining, cells were fixed with 4% paraformaldehyde, permeabilized with 0.02% Triton X-100 and blocked with 3% normal goat serum. Anti-mouse HSP60 or Calnexin (CNX) antibodies (both from BD Transduction Laboratories) were used at 1:5000 and 1:200 dilutions, respectively for 1 h at 37 °C, followed by incubation with Texas Red-conjugated goat-anti-mouse IgG (Invitrogen, 1:1000). Confocal images were captured with a spinning disk confocal microscope (WaveFx from Quorum Technologies) and an EMCCD camera (Hamamatsu, Japan). Representative images from at least three independent experiments are shown. Image analysis was performed using the Volocity (Improvision) software.

2.3. Purification of LDs

To isolate purified LDs, a sucrose gradient centrifugation method was adapted from [13]. Briefly, cells were homogenized and centrifuged at 10,000×*g* for 10 min to remove cell debris and crude mitochondria. One milliliter of post-mitochondria supernatant was mixed with equal volume of 2.5 M sucrose and overlaid with a discontinuous sucrose gradient composed of 400 μl each of 30%, 25%, 20%, 15%, 10% and 5% sucrose (w/v) prepared in homogenization buffer. The gradient was centrifuged at 166,000×*g* for 4 h at 4 °C in a SW55Ti rotor (Beckman Coulter) and seven fractions were collected from the top. Cell homogenates and gradient fractions were then analyzed by Western blotting using an anti-GFP antibody (Abcam).

3. Results

3.1. Lipin-1γ partially translocates from subcellular membranes to LDs upon fatty acid loading

Lipin-1α and β are localized differently in subcellular compartments. To characterize the subcellular localization of the γ isoform, we used a lipin-1γ-EGFP fusion protein expressed in COS-7 cells. In the absence of OA, the majority of lipin-1γ was present in a diffuse pattern representing the cytoplasm, with a small amount localized around LDs (Fig. 1A, top). The addition of OA/BSA induced the formation of numerous LDs, which was accompanied by lipin-1γ predominantly localizing around LDs (Fig. 1A, bottom). The same localization was also observed in HepG2 cells (not shown). To further confirm the LD localization of lipin-1γ, we purified LDs by sucrose gradient fractionation. Upon OA loading, a portion of lipin-1γ cofractionated with the LD-coat protein adipose differentiation-related protein (ADRP) in the purified LD fraction (Fig. 1B). However, a large amount of lipin-1γ co-fractionated with the ER marker protein CNX and the mitochondrial 60-kD heat shock protein (HSP60), possibly due to the limited capacity of LDs to accommodate large amount of overexpressed protein. However, we could not study the endogenous protein due to the lack of antibody against this specific isoform.

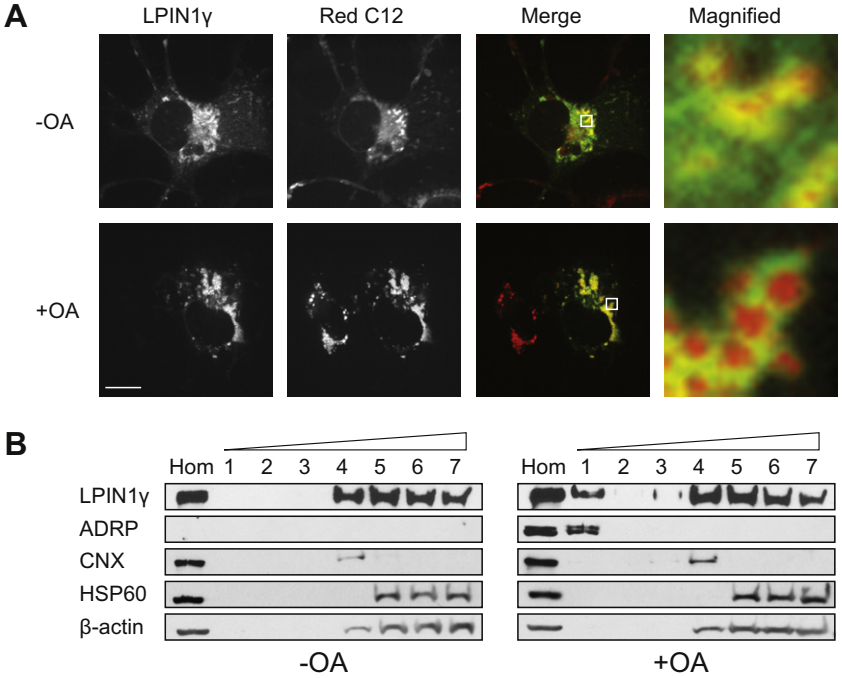

Fig. 1. Lipin-1γ is associated with LDs. (A) Localization of lipin-1γ on LDs by confocal imaging. COS-7 cells transiently transfected with lipin-1γ-EGFP plasmid (LIPN1γ) were incubated overnight in serum free DMEM containing Bodipy 558/568 C_{12} (Red C_{12}) in the absence (−OA) or presence of OA/BSA (+OA). Green, lipin-1γ; Red, LDs. Bar, 15 μm. (B) Lipin-1γ cofractionates with LDs. Cells expressing lipin-1γ-EGFP were homogenized and the post-mitochondrial supernatant was fractionated on a discontinuous sucrose gradient as describe in Section 2. The cofractionation of lipin-1γ with LD (ADRP), ER (CNX) and mitochondrial (HSP60) marker proteins was analyzed by Western Blotting. Hom, cell homogenate; CNX, calnexin. Figure shows the representative results from two independent experiments.

We further studied the potential association of lipin-1γ with subcellular compartments and demonstrated that it is mainly present in membrane fractions in the crude mitochondria/MAM (mitochondria-associated membranes) and microsomes, but not in the cytosol (Fig. 2A). The association with crude mitochondria/MAM was markedly reduced upon OA loading, accompanied with its appearance in the LD-associated fraction, suggesting a translocation from mitochondria/MAM to LDs. In contrast, no significant amount of the α-isoform was recovered in either fraction but the protein was instead mainly in microsomes and the cytosol. Confocal imaging further revealed that lipin-1 partially co-localized with HSP60 (Fig. 2B, arrow heads), suggesting its presence within or adjacent to mitochondria. In areas where lipin-1γ was enriched on LDs, HSP60 was largely absent (Fig. 2B, arrows), suggesting a preferential binding to LDs. Despite its presence in the microsomal fraction in subcellular fractionation (Fig. 1A), we did not observe considerable co-localization of lipin-1γ with CNX in the absence of OA, while the α-isoform exhibited partial co-localization (Fig. 2C).

3.2. The γ-specific domain is required but not sufficient for LD targeting

We further investigated the mechanism by which lipin-1γ is targeted to LDs. Hydrophobicity analysis of lipin-1γ primary sequence revealed that the γ-specific domain contains a stretch of hydrophobic amino acids, while the region immediately following it is distinctly hydrophilic (Fig. 3A). We constructed EGFP fusion proteins of the γ-specific domain (amino acid 536–561), the amphipathic region (γ-specific domain plus hydrophilic region, 536–601), as well as lipin-1α (which lacks the γ-specific domain, equivalent to γ-specific domain deletion) (Fig. 3B). All fusion proteins were expressed in the cells at expected sizes (Fig. 3C). Confocal microscopy showed that while full-length lipin-1γ assumed the expected localization around LDs, deletion of the γ-specific domain abolished this localization (Fig. 3D, LPIN1α). The expression of the γ-specific domain alone led to only a very small portion of the fusion protein around LDs and led to enlarged LDs, but the majority is diffusely localized in the cytoplasm (Fig. 3D, 536–561). Addition of the hydrophilic region completely restored LD-localization (Fig. 3D, 536–601).

3.3. Lipin-1γ expression alters morphology of LDs but not cell TG content

Proteins associated with LDs may affect LD morphology. Indeed, lipin-1γ expression in COS-7 cells led to smaller and clustered LDs, while non-transfected cells and cells expressing only EGFP contained mostly larger and dispersed LDs (Fig. 4A). Cells expressing lipin-1α also displayed dispersed LD distribution but the LDs were smaller (Fig. 4A). We further classified cells into three categories according to their LD morphology and distribution and quantified the number of cells under each category (Fig. 4B). Interestingly, despite the altered LD morphology, lipin-1γ expression did not affect cellular TG content while the α-isoform led to a small but significant increase in cell total TG (Fig. 4C). The increased TG content upon lipin-1α expression is consistent with previously reported observations [6]. These results suggest that TG level is not responsible for the altered LD morphology induced by lipin-1γ overexpression.

3.4. γ is the main lipin-1 isoform in human brain

To obtain some hint for the potential physiological relevance of lipin-1γ, we characterized mRNA expression of the three isoforms in selected human tissues, including brain, liver, small intestine,

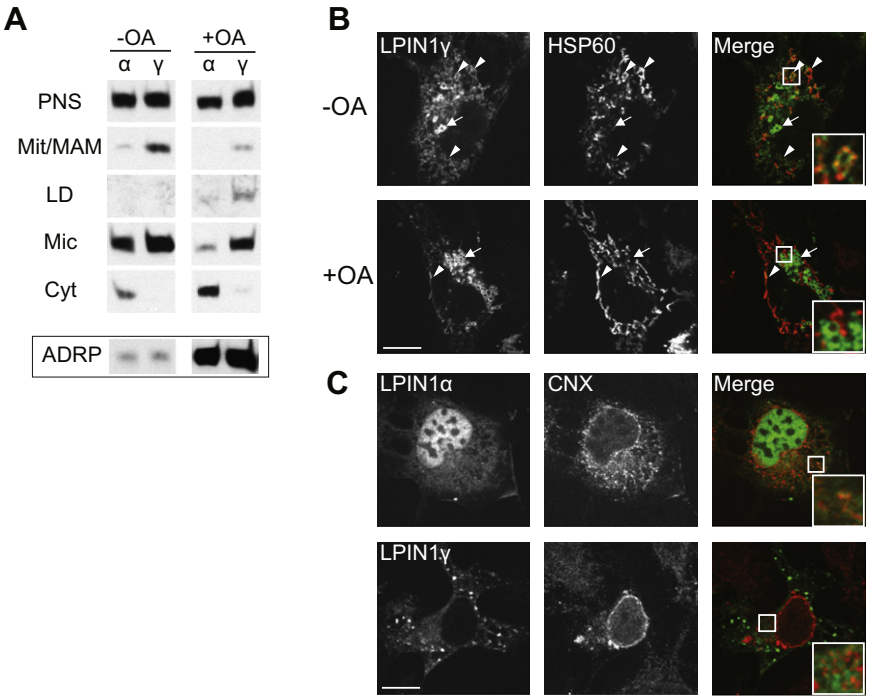

Fig. 2. Lipin-1γ is enriched in crude mitochondria and microsomes and translocates to LDs upon OA loading. (A) Association of lipin-1γ with subcellular compartments. Cells expressing lipin-1γ- or lipin-1α-EGFP were incubated overnight in the presence or absence of OA/BSA and subjected to subcellular fractionation described in Supplementary data. Enrichment of proteins in each fraction was detected by Western blotting. Equal volume from each fraction was analyzed. Panel in the box shows the level of ADRP in PNS. PNS, post-nuclear supernatant; Mit, mitochondria; MAM, mitochondria associated membranes; Mic, microsomes; Cyt, cytosol. Figure shows the representative results from three independent experiments. (B and C) Colocalization of lipin-1γ with mitochondria and the ER. Cells expressing lipin-1γ- or lipin-1α- EGFP were immunostained with anti-HSP60 (B) or calnexin (C) and analyzed by confocal microscopy. Arrowhead, area of colocalization; arrow, area where lipin-1γ is localized on LDs and excluded from HSP60. Bar, 15 μm.

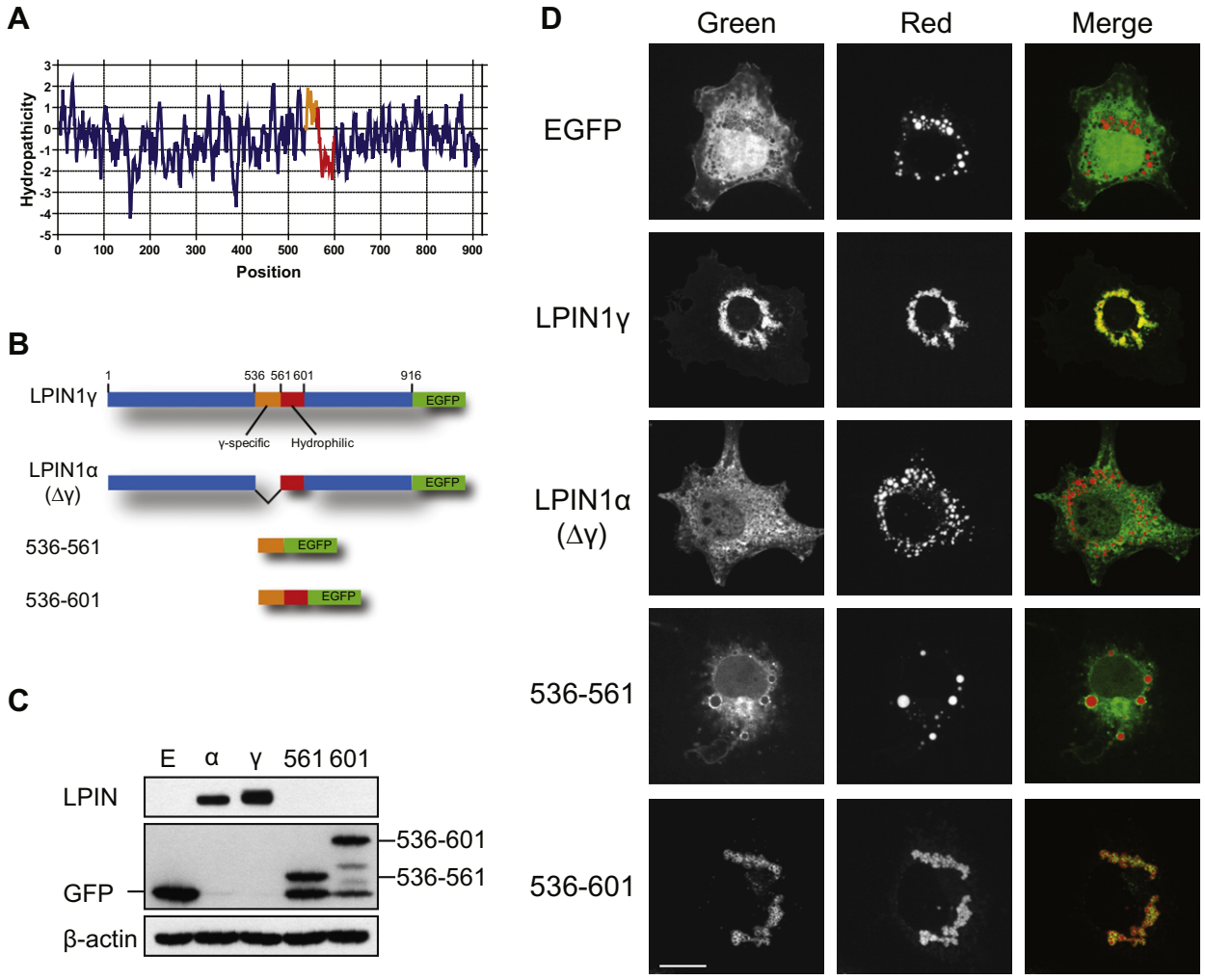

Fig. 3. The γ-specific region is required but not sufficient for LD targeting. (A) Hydrophobicity analysis of lipin-1γ primary sequence using the Kyte & Doolittle scale. Orange line, γ-specific region; red line, hydrophilic region immediately following the γ-specific region. (B) Scheme of the EGFP fusion proteins of full-length lipin-1γ and deletion mutants. (C) Expression of full-length lipin-1γ and deletion mutants. Lysates from cells expressing EGFP empty vector (E) and the EGFP fusion proteins were resolved by 4–20% SDS–PAGE and analyzed by Western blotting using anti-GFP. (D) Localization of the full-length and mutant proteins. Cells were incubated with OA/BSA overnight in the presence of Red C_{12} before imaging. Bar, 15 μm.

and skeletal muscle (Fig. 5A). LPIN1γ is expressed at a high level in the brain, but not in the liver and skeletal muscle where the other two isoforms are highly expressed [1]. Conversely, the expression of α- and β-isoforms in the brain was very low, suggesting that γ is the main brain isoform. Although tissues examined in our experiments are not exhaustive due to the availability of RNA samples, it is likely that lipin-1γ may be a brain-specific isoform. We further examined the expression of diacylglycerol acyltransferases (DGATs), enzymes converting DG to TG (subsequent to the step catalyzed by lipin-1). Only low levels of expression were detected when compared to other tissues, consistent with the previously published data. On the contrary, the levels of LD coat proteins, ADRP and TIP47, were comparable to those in the liver (Fig. 5B). These results imply that lipin-1γ may involve in LD formation in the brain but is less likely to provide precursors to DGATs.

4. Discussion

Lipin-1γ is a recently cloned isoform of lipin-1with unknown function. In this short article we report its preferential localization on LDs upon OA loading, despite the presence of an NLS signal known to direct the α-isoform into the nuclear. We demonstrated that the γ-specific region is required for its LD localization. To our knowledge this is the first protein whose LD-localization is regulated by mRNA alternative splicing. Interestingly, we also observed a translocation of lipin-1γ from the crude mitochondria to LDs upon OA treatment (Fig. 2). We speculate that lipin-1γ may be localized at the LD-mitochondria junction or MAM before cells forming large amounts of LDs, and is recruited to LDs when excessive fatty acids are present. Unfortunately we were only able to study the localization based on overexpression of tagged proteins due to the lack of isoform-specific antibodies. Even though we were able to reproduce the same localization in HepG2 cells (not shown), it will be intriguing to study localization and function of this protein at an endogenous level.

The expression of lipin-1γ induced an interesting phenotype in LDs, leading to smaller and clustered LDs without changing total cell TG, possibly due to increased PAP activity which may result in reduced PA and accumulation of DG around LDs. PA and DG are increasingly recognized to play important roles in LD formation. It is recently found that deletion of Pah1, the yeast homologue of lipin-1, leads to larger LDs in yeast due to the accumulation of the fusogenic PA (Yang, H. and Walther, T.C., unpublished observa-

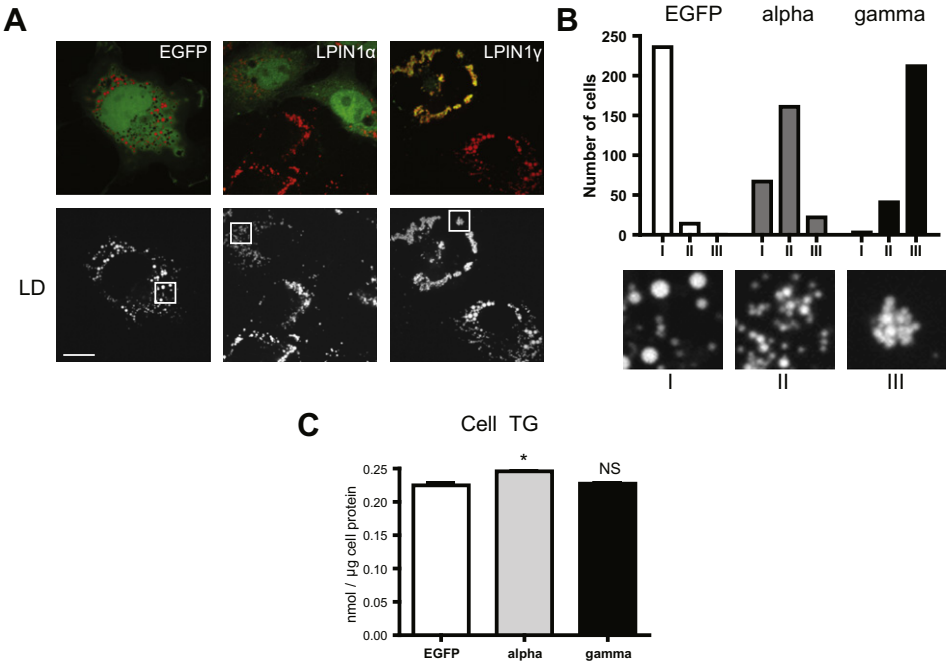

Fig. 4. Expression of lipin-1γ alters lipid droplet morphology but does not affect cellular TG. (A) Confocal images of LDs in cells expressing EGFP, lipin-1α-EGFP and lipin-1γ-EGFP. Bar, 15 μm. (B) Classification of LD morphology. Cells were grouped into three categories according to LD morphology: I, big and dispersed; II, small and dispersed; III, small and clustered. A total of 250 cells of each transfectant were analyzed and results from three independent experiments are presented. Micrographs show the representative LD morphology from each category. (C) Cell homogenates were analyzed for total cellular TG (described in Supplementary data) normalized to total cell protein. ∗, $P < 0.01$.

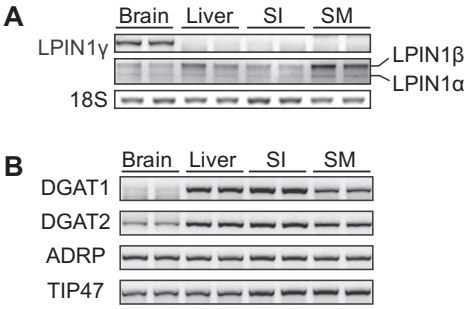

Fig. 5. Lipin-1γ is the main isoform in human brain. (A) mRNA expression of the three lipin-1 isoforms in human tissues. RT-PCR was performed as described in Supplementary data using total RNAs of normal human brain, liver, small intestine (SI) and skeletal muscle (SM) using primers described in Table S1. (B) Expression of DGATs, ADRP and TIP47 in normal human tissues.

tions). Another independent study also reported that Pah1 deletion caused morphological change in LDs due to decreased DG level [14].

Furthermore, we discovered that the lipin-1γ isoform is not expressed in apolipoprotein B-expressing tissues (liver and intestine), nor in the skeletal muscle, unlike other lipin-1 isoforms, but is almost exclusively in the brain (Fig. 5A). The physiological significance of LDs in the brain is unclear and the presence of LDs has only been reported in neurons cultured in vitro. Our research demonstrated that LD coat proteins ADRP and TIP47 are expressed at comparable levels in the brain (Fig. 5B), suggesting that normal human brain is able to accumulate LDs. A series of other enzymes governing glycerolipid synthesis have been found expressed in the brain [15]; peculiarly, AGPAT4 is expressed at a very high levels, suggesting that brain has the capacity to synthesis glycerolipids at a relatively high rate and that these enzymes may work in concert to maintain lipid homeostasis in this organ. Since DG produced by PAP activity can either be used for TG synthesis or PL synthesis, it is likely that different lipin-1 isoforms direct the DG product for different metabolic fates. The expression of lipin-1γ led to smaller LDs (Fig. 4B) but no change in total cellular TG (Fig. 4C), suggesting increased surface area of LDs, and hence increased PL/TG ratio. Thus we speculate that lipin-1γ may be specialized to provide DG for PL synthesis instead of TG. This process may be especially important for regulating brain functions that require large amounts of PLs. However, further investigation is needed to understand the composition, function and regulation of brain LDs.

Acknowledgements

We thank Dr. John Brumell and Dr. Peter Kijun Kim (The Hospital for Sick Children) for providing the eGFP-N1 vector and the HSP60 antibody, respectively. H.W. is supported by fellowships from CIHR Training Program in Protein Folding, as well as Heart & Stroke/Richard Lewar Centre of Excellence (University of Toronto). This work was supported by an operating grant (T-6041) from the Heart and Stroke Foundation of Ontario to K.A. and the National Institutes of Health grant GM-28140 to G.M.C.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.05.035.

References

[1] Donkor, J., Sariahmetoglu, M., Dewald, J., Brindley, D.N. and Reue, K. (2007) Three mammalian lipins act as phosphatidate phosphatases with distinct tissue expression patterns. J. Biol. Chem. 282, 3450–3457.
[2] Han, G.S., Wu, W.I. and Carman, G.M. (2006) The *Saccharomyces cerevisiae* Lipin homolog is a Mg^{2+}-dependent phosphatidate phosphatase enzyme. J. Biol. Chem. 281, 9210–9218.
[3] Langner, C.A., Birkenmeier, E.H., Ben-Zeev, O., Schotz, M.C., Sweet, H.O., Davisson, M.T. and Gordon, J.I. (1989) The fatty liver dystrophy (fld) mutation.

A new mutant mouse with a developmental abnormality in triglyceride metabolism and associated tissue-specific defects in lipoprotein lipase and hepatic lipase activities. J. Biol. Chem. 264, 7994–8003.
[4] Peterfy, M., Phan, J., Xu, P. and Reue, K. (2001) Lipodystrophy in the fld mouse results from mutation of a new gene encoding a nuclear protein, lipin. Nat. Genet. 27, 121–124.
[5] Phan, J. and Reue, K. (2005) Lipin, a lipodystrophy and obesity gene. Cell Metab. 1, 73–83.
[6] Bou Khalil, M. et al. (2009) The level and compartmentalization of phosphatidate phosphatase-1 (lipin-1) control the assembly and secretion of hepatic VLDL. J. Lipid Res. 50, 47–58.
[7] Chen, Z., Gropler, M.C., Norris, J., Lawrence Jr., J.C., Harris, T.E. and Finck, B.N. (2008) Alterations in hepatic metabolism in fld mice reveal a role for lipin 1 in regulating VLDL-triacylglyceride secretion. Arterioscler. Thromb. Vasc. Biol. 28, 1738–1744.
[8] Han, G.S. and Carman, G.M. (2010) Characterization of the human LPIN1-encoded phosphatidate phosphatase isoforms. J. Biol. Chem. 285, 14628–14638.
[9] Peterfy, M., Phan, J. and Reue, K. (2005) Alternatively spliced lipin isoforms exhibit distinct expression pattern, subcellular localization, and role in adipogenesis. J. Biol. Chem. 280, 32883–32889.
[10] Peterfy, M., Harris, T.E., Fujita, N. and Reue, K. (2010) Insulin-stimulated interaction with 14-3-3 promotes cytoplasmic localization of lipin-1 in adipocytes. J. Biol. Chem. 285, 3857–3864.
[11] Harris, T.E., Huffman, T.A., Chi, A., Shabanowitz, J., Hunt, D.F., Kumar, A. and Lawrence Jr., J.C. (2007) Insulin controls subcellular localization and multisite phosphorylation of the phosphatidic acid phosphatase, lipin 1. J. Biol. Chem. 282, 277–286.
[12] Huffman, T.A., Mothe-Satney, I. and Lawrence Jr., J.C. (2002) Insulin-stimulated phosphorylation of lipin mediated by the mammalian target of rapamycin. Proc. Natl. Acad. Sci. USA 99, 1047–1052.
[13] Ingelmo-Torres, M. et al. (2009) Hydrophobic and basic domains target proteins to lipid droplets. Traffic 10, 1785–1801.
[14] Adeyo, O., Horn, P.J., Lee, S., Binns, D.D., Chandrahas, A., Chapman, K.D. and Goodman, J.M. (2011) The yeast lipin orthologue Pah1p is important for biogenesis of lipid droplets. J. Cell Biol. 192, 1043–1055.
[15] Takeuchi, K. and Reue, K. (2009) Biochemistry, physiology, and genetics of GPAT, AGPAT, and lipin enzymes in triglyceride synthesis. Am. J. Physiol. Endocrinol. Metab. 296, E1195–E1209.